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A critical role of STAT1 in streptozotocin-induced diabetic liver injury in mice: Controlled by ATF3☆

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ABSTRACT

It is well-established that the administration of streptozotocin accelerates diabetic liver injury as well as type-I diabetes, however the underlying mechanisms are poorly understood. Here we investigated the molecular mechanisms of diabetic liver injury in a model of streptozotocin (STZ)-induced type-I diabetes. STZ administration induced type-1 diabetes and chronic liver injury was associated with increased STAT1, which is implicated in diabetic liver injury by virtue of its ability to promote hepatocyte apoptosis, in the liver and pancreas, which were all strongly inhibited in STAT1$^{-/-}$ mice. Similarly, STZ-induced ATF3, a stress-inducible gene, was completely abolished in the liver of IFN-γ$^{-/-}$ mice, but not in STAT1$^{-/-}$ mice. Inhibition of STAT1 by siRNA or dominant-negative DNA did not affect ATF3 protein expression but blocked IFN-γ-induced ATF3 translocation from the cytosol into the nucleus. In contrast, inhibition of ATF3 by using siRNA diminished STAT1 protein expression and IFN-γ/STZ-induced hepatocyte apoptosis. Furthermore, GST pull-down and co-IP assay showed that STAT1 bound to C-terminal domain of ATF3. Such direct interaction increased the stability of STAT1 by inhibiting its ubiquitination as well as proteasome activity. Our results suggest that STAT1 is a common signaling pathway contributing to STZ-induced diabetes and diabetic liver injury. ATF3 functions as a potent regulator of STAT1 stability, accelerating STZ-induced diabetes and diabetic liver injury.

1. Introduction

Epidemiological studies show that diabetic patients are at higher risk of chronic liver disease and hepatocellular carcinoma [1–4]. Among them, non-alcoholic fatty liver disease (NAFLD) is probably the most prevalent liver disorder in type-II diabetes that is usually accompanied by central obesity, insulin resistance and other metabolic abnormalities although whether or how NAFLD and insulin resistance are temporally and mechanistically related is controversial [5–7]. Diabetes and insulin resistance were also identified as important factors associated with an increased risk of advanced liver fibrosis in patients with normal ALT [3,8]. The insulin resistance-associated inflammation, lipogenesis, and fibrogenesis are important mechanisms contributing to the pathogenesis of chronic liver disease in type-II diabetes [6,9,10]. In addition, type-I diabetes is also associated with increased risk of chronic liver injury [11,12], however the underlying mechanisms remain largely unknown.

Administration with streptozotocin (STZ) is a well-established experimental tool for the induction of autoimmune type-I diabetes, which is mediated by hyperglycemia and hypoinsulinemia due to its selective cytotoxicity towards insulin-secreting pancreatic β-cells [13,14]. Interestingly, STZ administration also causes diabetic organ complications in various tissues including the liver, kidney, heart, and brain as well as the pancreas [15–17]. In STZ-administrated mice or rat, cytokines secreted through T-cell activation and hyperglycemia as independent risk factors directly cause liver damage, lending to diabetic liver injury. Although the molecular mechanisms underlying STZ-mediated pancreatic β-cell death have been extensively investigated, how STZ induces diabetic liver injury is still not fully understood. Increasing evidence suggests that activation of IFN-γ/STAT1 plays an essential role in STZ-mediated pancreatic β-cell apoptosis and diabetes

Abbreviations: STAT1, signal transducer and activator of transcription factor 1; STZ, streptozotocine; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

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because STAT1$^{-/-}$ mice are resistant to multiple low-dose STZ-induced diabetes and β-cell apoptosis [18,19]. Recently, others and we have demonstrated that STAT1 activation by IFN-γ also plays an important role in liver injury and hepatocyte apoptosis in a variety of liver injury models induced by LPS/D-GalN and concanavalin A [20–23]. Thus, we hypothesized that injection of STZ can activate IFN-γ/STAT1 signaling pathway, which subsequently not only induces pancreatic β-cell apoptosis but also induces hepatocyte apoptosis, resulting in type-I diabetes and diabetic liver injury, respectively.

ATF3, a member of the ATF/cAMP-responsive element binding protein subfamily, is a stress-inducible transcriptional repressor as well as a basic region-leucine zipper transcription factor [24]. However, the findings about the role of ATF3 in gene regulation and apoptosis have been controversial [25,26]. Activated ATF3 can homodimerize and repress transcription of various promoters with ATF sites or heterodimerize with bZip proteins, c-jun, Jun B, ATF2, or Gadd153/CHOP10 (C/EBP homologous protein) and activate transcription of target genes [27,28]. Recently, we have reported that ATF3 negatively regulates adiponectin gene expression in obesity and type-II diabetes and that lipotoxicity-increased ATF3 was involved in ROS production and loss of mitochondria membrane potential, resulting in pancreatic β-cell apoptosis [29,30].

In this paper, we demonstrated that during STZ-mediated diabetic liver injury, elevated ATF3 interacted directly with STAT1 and enhanced the stability of the latter, and therefore it may serve as a liver injury-inducing factor via stimulation of the apoptotic functions of STAT1.

2. Materials and methods

2.1. Mice

Eight-week-old male STAT1$^{-/-}$ on 129S6/SvEv background and 129S6/SvEv wild-type mice were originally purchased from Taconic (Germantown, NY). BALB/c-background IFN-γ$^{-/-}$ and BALB/c wild-type mice were purchased from Jackson Laboratory (Bar Harbor, ME). For STZ injection, the mice were injected intraperitoneally for 5 consecutive days with STZ (80 mg/kg; sigma) freshly dissolved in 50 mM sodium citrate buffer (pH 4.5) and sacrificed 14 days post the final STZ injection. Animals were considered as diabetics when blood glucose levels exceeded 250 mg/dl, usually within 7 days from the final injection. Furthermore, anti-IFN-γ (2 μg/200 μl) was i.v. injected into mice. All animal experiments were conducted in accordance with guidelines from the Korean National Institutes of Health Animal Facility.

2.2. Plasmids

Human wild-type ATF3 and mutated ATF3 (ΔC, 1-100) with a C-terminal deletion, cDNA expression vectors were a generous gift from Dr. T Hai (Ohio State University). Human ATF3 and ATF3(ΔC) cDNA were amplified separately by PCR and cloned into pEGFP-C2 and pGEX-4T-1 vectors (Clontech, Mountain View, CA). The pcDNA3-Flag-STAT1 or -DN-STAT1 constructs were generously provided by Dr. B. Gao (NIH/NIAAA). From pcDNA3-Flag-STAT1, GFP-STAT1 was constructed by subcloning into the BamHI/XbaI restriction sites of pEGFP-C1.

2.3. Isolation of primary mouse hepatocytes and islet cells

Mice weighing 20–25 g were anesthetized with pentobarbital sodium (30 mg/kg, IP), and the isolation of primary hepatocytes by using liver perfusion method was followed as described previously [22]. Next, islet cells were isolated from overnight-fasted C57BL/6 mice by the collagenase digestion technique as described previously [39].

2.4. RT-PCR analysis

RT-PCR analysis and the sequences of the primers used for murine IFN-γ and TNF-α are as follows: IFN-γ, (F) 5′-AACGCTACACACTGCATC-3′ and (R) 5′-AGCTCATTGAATGCTTGG-3′; TNF-α, (F) 5′-GGCAGGTC-TACTTTGGAGTCA TTGC-3′ and (R) 5′-ACATTCGAGCCAGTGAATTCGG-3′.

2.5. Immunoassaying

Immunohistochemistry and immunocytochemistry analysis were followed as described previously [39].

2.6. GST pull-down assay

Five hundred microgram of lysates was incubated with 3.0 μg of GST or GST-ATF3 proteins coupled to glutathione Sepharose beads in 300 μL of lysis buffer overnight at 4 °C with continuous rocking as described previously [22].

2.7. Proteasome assays

Cells were transfected with Neo-vector, ATF3, or ATF3(ΔC) cDNA. For 20S proteasome inhibition assays, 5 mM Suc-LLVY-AMC substrates and inhibitor, epoxomicin, which is a rapid, potent and irreversible inhibitor of 20S proteasome chymotrypsin-like activity, in DMSO were added to assay solutions at a final DMSO concentration of 1%. The following assay buffer was used: 20 mM Tris–HCl, pH 8.0, 0.5 mM EDTA (plus 0.035% SDS for Suc-LLVY-AMC assays). 20S proteasome was added to the assay buffer containing substrates, inhibitors, and test sample at a final volume of 100 mL at room temperature (25 °C) in a Dynex (Chantilly, CA) Microfluor II 96-well plate and the fluorescence emission immediately was measured at 460 nm (lex, 360 nm) by using a Cytofluor (Perspective Biosystems, Framingham, MA) fluorescence plate reader for 50 min [31].

2.8. In vitro and in vivo ubiquitination assays

In vitro ubiquitination assays were performed as described previously [27] with some modifications. Recombinant purified GST-STAT1 was preincubated with 250 ng of recombinant full-length or the deleted ATF3 protein at 37 °C for 1 h in a 30 μL reaction buffer 1 containing 50 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM DTT, 4 mM ATP, 100 μM E1, 10 μM E2, 5 μg His-ubiquitin (E1,E2, and His-ubiquitin, Boston Biochem) and His-ubiquitinated proteins were isolated by incubating at 4 °C for 1 h with 20 μL of Ni-nitrilotriacetate agarose (Qiagen) in a final volume of 200 μL in reaction buffer 2 containing 50 mM sodium phosphate, pH 7.9, 300 mM NaCl, 0.05% Tween 20, and 10 mM imidazole. After low-speed centrifugation (735×*g*), washed Ni-agarose beads containing His-ubiquitinated proteins were eluted and then subjected to Western blotting for ubiquitinated STAT1 using anti-polyubiquitin antibody. *In vivo* ubiquitination assay was also performed as described previously [26]. Cells were co-transfected with the constant amount of HA-STAT1 (0.5 μg) and His-ubiquitin (0.5 μg), together with Flag-ATF3 or ATF3 (ΔC) (0.5 μg). Forty-eight hours after transfection, cells were treated with 10 μM MG-132 for 6 h before being harvested. His-ubiquitin-containing protein complexes were pulled down with Ni-agarose beads, and subsequently resolved by 10% SDS-PAGE, followed by immunoblotting with anti-ubiquitin, anti-HA or anti-Flag antibodies.

2.9. Statistical analysis

For comparing values obtained in three or more groups, one-factor analysis of variance was used, followed by Tukey's post hoc test, and $P<0.05$ was taken to imply statistical significance.